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Vaccine 19 (2001) 4072–4080

Vaccine

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Intranasal immunisation with influenza-ISCOM induces strong mucosal as well as systemic antibody and cytotoxic T-lymphocyte responses

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Received 25 October 2000; received in revised form 5 February 2001; accepted 5 March 2001

Abstract

Intranasal administration of vaccines is preferred for induction of mucosal immune responses. In this study, mice were immunised intranasally and subcutaneously with influenza-immuno stimulating complexes (influenza-ISCOM). The intranasal dose was 15-times the subcutaneous dose. All mice dosed with influenza-ISCOMs survived challenge with live virus and comparable serum antibody and splenic cytotoxic T-lymphocyte responses were detected in both groups. Induction of mucosal IgA was significantly higher with intranasal immunisation and was comparable to responses induced with the heat labile enterotoxin of *Escherichia coli* as adjuvant. These findings demonstrate that intranasal administration of high dose influenza-ISCOM results in potent systemic and mucosal immune responses.

Keywords: Influenza-ISCOM; Intranasal administration; Mucosal immune response

1. Introduction

Many natural infections caused by microorganisms are initiated by invasion at mucosal surfaces. It is likely that immunisation against these microorganisms is dependent on induction of potent mucosal immune responses and therefore vaccines capable of inducing such responses are needed [1,2]. To date, very few mucosally administered vaccines have been approved for human use, and those that have, including poliomyelitis and Salmonella typhi, are based on live attenuated microorganisms.

Vaccine induction of immune responses via mucosal routes is generally less efficient than the parenteral route and varies depending on both the route of administration (intranasal, oral, rectal or vaginal) and the formulation, in particular the adjuvant [2]. The most powerful mucosal adjuvants identified to date are the toxin from *Vibrio cholerae* (CT) and the heat labile enterotoxin of *Escherichia coli* (LT) [1–4]. Both have been shown to induce high levels of secretory IgA, however their toxicity precludes their use in human vaccines. Much effort has been directed at generating non-toxic mutant forms of LT and CT, which retain adjuvant activity. For example LT-K63, a non-toxic, intact LT holotoxin with an unmodified β subunit able to bind GM1 gangliosides has been shown in mice to induce enhanced mucosal IgA responses to a number of viral antigens including influenza [5]. Other candidate mucosal adjuvants include the hematopoietic growth factor GM-CSF [6], DNA or synthetic oligodeoxynucleotides containing immunostimulatory CpG motifs and ISCOM (immuno stimulating complex, ISCOM® is a registered trademark of ISCOTEC AB, Sweden) [7–10].

ISCOMs are typically 40 nm spherical cage-like particles formed by a combination of *Quillaia* saponin, cholesterol, phospholipid and antigen. ISCOMs have been shown, using a variety of antigens, to induce potent humoral and cellular immune responses, includ-

Abbreviations: ISCOM – immuno stimulating complex, ISCOM® is a registered trademark of ISCOTEC AB, Sweden.

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0264-410X/01/$ - see front matter
PII: S0264-410X(01)00110-4

ing enhanced cytokine secretion and activation of cytotoxic T-lymphocyte (CTL) responses [9,10]. Intranasal delivery of ISCOMs containing antigens from a variety of sources including influenza [11,12], measles [13], Echinococcus granulosus [14,15], herpes simplex type 2 [16] and respiratory syncytial virus [17] have been shown to induce secretory and serum IgA as well as CTL and provide protection against infection.

A common way of improving the response to mucosally administered vaccines has been to increase the dose of antigen. In this study, we have investigated the effect on the mucosal and systemic immune responses, as well as efficacy, of increasing the dose of both antigen and adjuvant of an intranasally administered ISCOM vaccine prepared with disrupted A/PR8/34 influenza virus antigen (influenza-ISCOM).

2. Materials and methods

2.1. Preparation of influenza-ISCOM

Influenza-ISCOMs were prepared as previously described [18]. Briefly, to a solution of disrupted A/PR8/34 virus was added a mixture of *Quillaia* saponin fractions (ISCOPREP®703, ISCOTEC AB), cholesterol (Sigma, USA) and di-palmitoyl phosphatidyl choline (Avanti, USA) dissolved in the detergent MEGA-10 (Sigma). The reaction mixture was stirred and then extensively dialysed against PBS. Formation of ISCOM was confirmed by electron microscopy and the level of ISCOPREP® was determined using HPLC.

2.2. Immunisations

BALB/c mice, 10–12 week old, were immunised either subcutaneously with 100 μl into the scruff of the neck or intranasally, after being anaesthetised, by placing 30 μl onto the nares and allowing mice to inhale. All mice remained healthy throughout the study.

For efficacy studies, mice were challenged five weeks after one immunisation with either intranasal or subcutaneous influenza-ISCOMs or non-adjuvanted influenza virus (Table 1). Doses of influenza antigen and adjuvant (ISCOPREP®703) in the subcutaneous ISCOM group were selected to give 100% survival and were based on previous experience [19]. Mice in the intranasal ISCOM group received a 15 times higher dose of both influenza antigen and adjuvant (ISCOPREP™703). A control group was immunised intranasally with PBS.

For immunological studies mice were immunised twice, four weeks apart, as described above with an additional group being immunised intranasally with influenza antigen adjuvanted with 5 μg/dose of heat labile enterotoxin of *E. coli* (LT, Sigma, St. Louis). The dose of LT was typical of that used by others to induce responses [20]. Dose details are summarised in Table 2. Mice were bled seven days post the second immunisation and serum analysed for antibody.

2.3. Collection of mucosal samples

Mice were euthanased under CO_2 eight days post the second immunisation. The trachea was exposed in the neck and a cannula inserted towards the lungs. Using a 1-ml syringe, PBS (1 ml) was washed in and out of the lungs three times and then added to tubes containing 10 μl of 100 × protease inhibitor cocktail (PIC, containing 100 μM Pepstatin A, 1 mM Leupeptin, 20 mM Pefabloc SC (AEBSF)). The cannula was then removed and re-inserted towards the nasal cavity. Using a 1-ml syringe, PBS (0.75 ml) was washed twice through the nasal area and 7.5 μl of the PIC solution added. The lung and nasal washes were then centrifuged at 10000 × *g* for 5 min and 0.5 ml of the supernatant was collected. PBS (50 μl) containing 5 μl of 10 × PIC and 5% FBS was added prior to storage at −20°C.

For faecal antibody determination, four faecal pellets were collected from each mouse and added to PBS (1 ml) containing 5 μl of 100 × PIC. The pellets were vortexed vigorously to break them up and then centrifuged at 2000 × *g* for 5 min to remove solid matter. The supernatant was removed and stored at −20°C.

Table 1
Outcome of challenge with influenza virus after immunisation with influenza-ISCOMs or with non-adjuvanted influenza virus

Formulation	Immunisation route	Influenza (μg/dose)	ISCOPREP®703 (μg/dose)	Survival (%)	Weight gain on day 5 (%)
Influenza-ISCOM	i/n	0.15	25	100	4.4
Influenza-ISCOM	s/c	0.01	1.7	100	4.7
Influenza	i/n	0.05	–	10	−16.0
Influenza	i/n	0.15	–	10	−19.0
Influenza	i/n	1.5	–	30	−15.3
Influenza	i/n	5	–	100	0.5
Control	i/n	–	–	0	−21.5

Table 2
Immunisation with influenza-ISCOMs or influenza antigen mixed with LT[a]

Formulation	Immunisation route	Influenza (μg/dose)	Adjuvant (μg/dose)
Influenza-ISCOM	i/n	1.5	90
Influenza-ISCOM	s/c	0.1	6
Influenza+LT	i/n	1.5	5
Control	i/n	–	–

[a] Groups were analysed for induction of influenza specific serum and mucosal antibody responses as well as splenic CTL (not the group immunised with Influenza+LT).

2.4. Challenge procedure

Challenge was performed according to the method described by Coulter et al. [19]. Briefly, immunised mice were challenged with airborne homologous live influenza virus of the mouse adapted pathogenic strain A/PR8/34 seven days post the second immunisation. Mice were exposed over a period of 10 min to $10^{7.3}$ tissue culture infectious doses ($TCID_{50}$) per ml of influenza virus. The outcome was monitored by weight change five days after the challenge and survival nine days later.

2.5. Enzyme immunoassay (EIA)

Antibody to whole influenza virus in serum, faecal samples and lung and nasal washes was assayed by a standard indirect enzyme immunoassay [19]. Briefly, plates were coated by overnight incubation with 10 μg/ml of purified MDCK grown A/PR8/34 virus, followed by blocking in PBS containing 1% casein. Sera and mucosal samples were tested at five-fold dilutions starting from 1:100 or 1:5. The plates were incubated with HRP conjugated anti mouse IgG (KPL, USA) or IgA (KPL), followed by the addition of tetramethylbenzidine (TMB) substrate solution (KPL). The reaction was stopped by addition of H_2SO_4, and the OD read at 450 nm. Titres were determined from a standard curve generated on each plate using four-parameter fit calculations (KCJr, Bio-Tek Instruments, USA).

2.6. Cytotoxic T-lymphocyte assay

Influenza antigen specific cytotoxic T-cell responses (CTL) were assayed using in vitro restimulated spleen cell cultures generated from vaccinated and control BALB/c mice according to the method described by Coulter et al. [19]. Spleen cells at 2.5×10^6/ml were cultured in 24 well plates (Costar, USA) together with 0.5×10^6/ml in vitro infected (influenza A/PR8/34) spleen cells from naive mice. Effector cells were cultured for five days at 37°C, in RPMI 1640 medium (CSL Limited, Australia) containing (5% CO_2), 10% v/v inactivated foetal bovine serum (FBS, CSL Limited), 5×10^{-5} M 2-mercaptoethanol (Gibco BRL, USA) and 40 μg/ml gentamicin (CSL Limited). The cells were then recovered, washed, counted and mixed with either 10^4 uninfected (negative target cells) or A/PR8/34-virus infected and ^{51}Cr-labelled P815 cells (positive target cells) together with a 20 fold excess of uninfected unlabelled P815 cells (cold target inhibition of natural killer-like cytotoxic activity) in 96 well plates (Nunc, 0.2 ml of RPMI 1640 containing 10% FBS) for 4 h at 37°C in 5% CO_2 at the indicated E:T ratios. The plates were centrifuged and the supernatant was sampled and analysed for released ^{51}Cr. The percentage specific ^{51}Cr-release was calculated as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release was determined from target cells incubated without the addition of effector cells and maximum release was determined from cells lysed by the addition of 0.1% Triton X100 (Sigma). Spleen cells from mice infected intraperitoneally with 50 μg of haemagglutinin as live A/PR8/34 influenza virus were used as positive control. A result was considered positive, when the specific ^{51}Cr-release was 10% higher than the spontaneous ^{51}Cr-release.

2.7. Statistics

Titres, expressed as individual titres and geometric mean titres, were compared with respect to the incidence and level of antibody by the Mann–Whitney U-test, using InStat software (v3.0, GraphPad, USA). P values < 0.05 were considered to represent statistically significant differences.

3. Results

3.1. Challenge with influenza virus after immunisation with influenza-ISCOM

Mice immunised subcutaneously or intranasally with influenza-ISCOMs were challenged with live airborne influenza virus. Five days after the challenge all mice in the two ISCOM groups immunised with 0.01 or 0.15 μg of influenza had survived and had gained approximately 4–5% in weight (Table 1). In contrast, all mice in the control group died, having previously lost 20% of their weight.

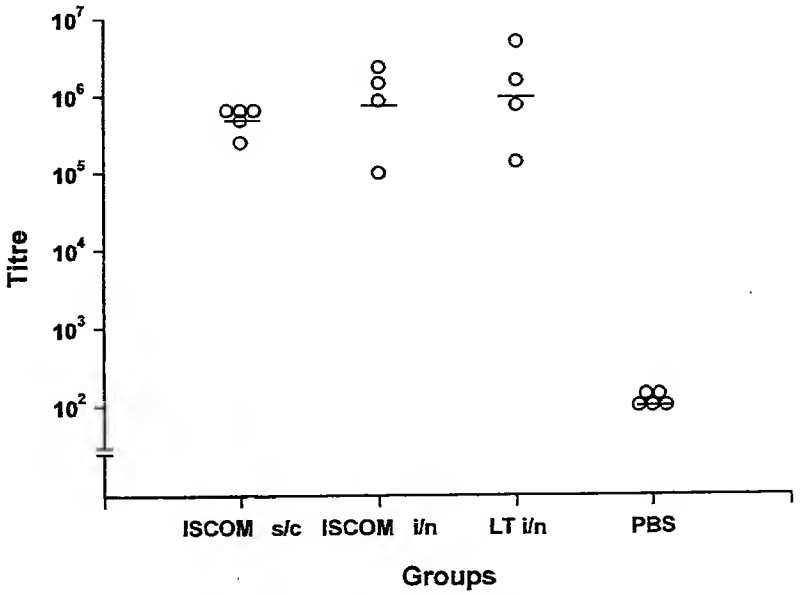

Fig. 1. Analysis of influenza specific IgG responses in serum after immunisation with the different experimental vaccines detailed in Table 2. Individual titres (O) and geometric mean titres (–) are shown after two immunisations.

Mice immunised intranasally with non-adjuvanted disrupted influenza virus at levels up to 1.5 μg of influenza virus lost more than 15% of their weight and showed poor survival rates (30% in the 1.5 μg group). However, when the dose was increased to 5 μg mice gained weight (0.5%), and all mice in this group survived.

3.2. Serum antibody response to ISCOM and LT adjuvanted influenza virus

Mice in both ISCOM (subcutaneous and intranasal) and LT adjuvanted influenza-groups demonstrated high titres of influenza specific serum IgG with no significant differences ($P < 0.05$) between groups (Fig. 1). Influ-

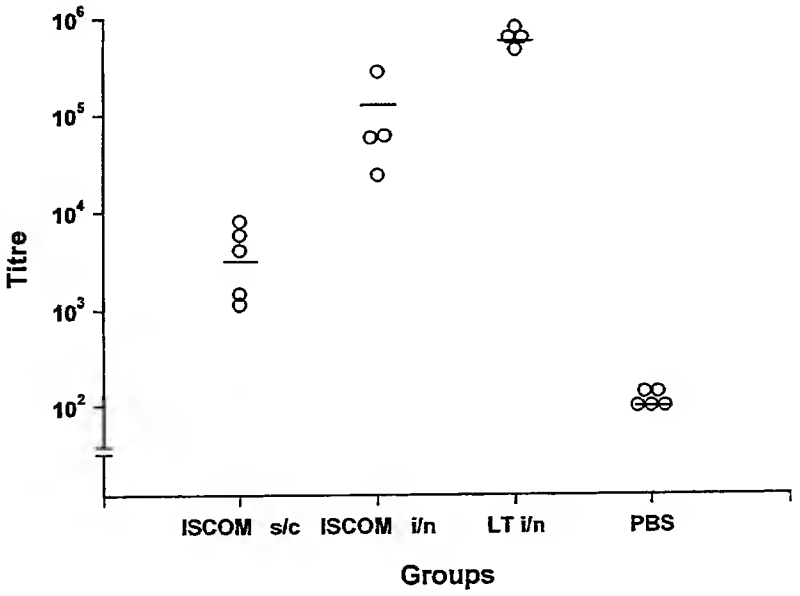

Fig. 2. Analysis of influenza specific IgA responses in serum after immunisation with the different experimental vaccines detailed in Table 2. Individual titres (O) and geometric mean titres (–) are shown after two immunisations.

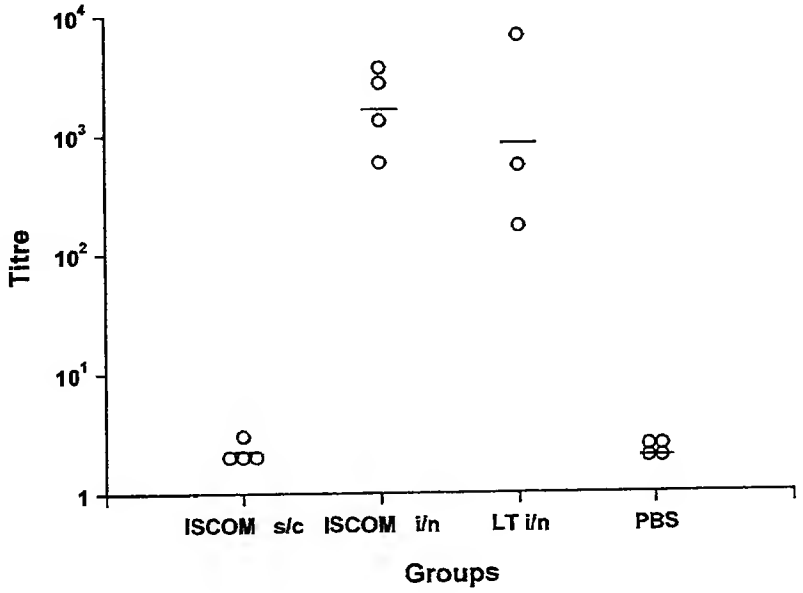

Fig. 3. Analysis of influenza specific IgA responses in nasal washes after immunisation with the different experimental vaccines detailed in Table 2. Individual titres (O) and geometric mean titres (–) are shown after two immunisations.

enza specific IgA in serum was significantly higher in the group immunised intranasally with influenza-ISCOMs compared with subcutaneous administration ($P < 0.05$) (Fig. 2). The serum IgA response induced in the group immunised with influenza antigen mixed with LT was significantly higher than in both influenza-ISCOM groups ($P < 0.05$).

3.3. Mucosal antibody response to ISCOM and LT adjuvanted influenza virus

Similar high levels of influenza specific IgA were detected in nasal washing (Fig. 3) and lung (Fig. 4) from mice immunised intranasally with influenza-ISCOMs or influenza antigen mixed with LT. In contrast,

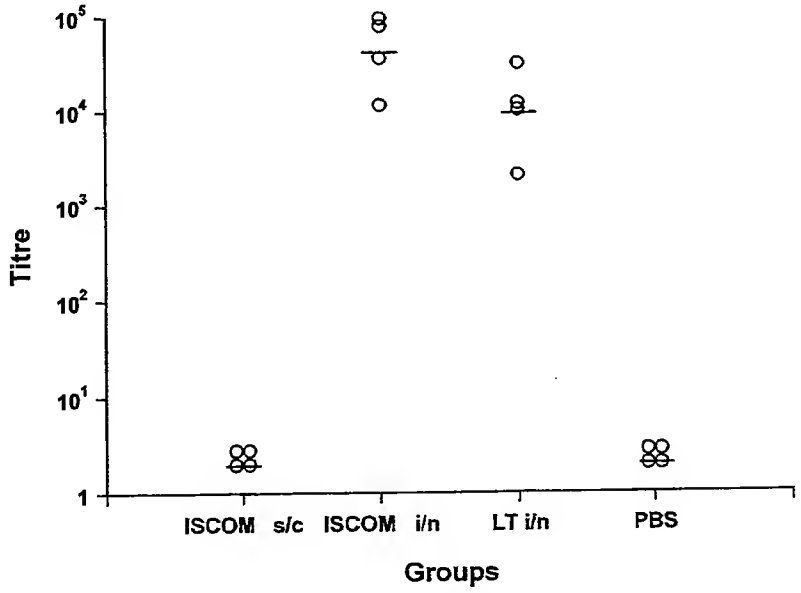

Fig. 4. Analysis of influenza specific IgA responses in lung washes after immunisation with the different experimental vaccines detailed in Table 2. Individual titres (O) and geometric mean titres (–) are shown after two immunisations.

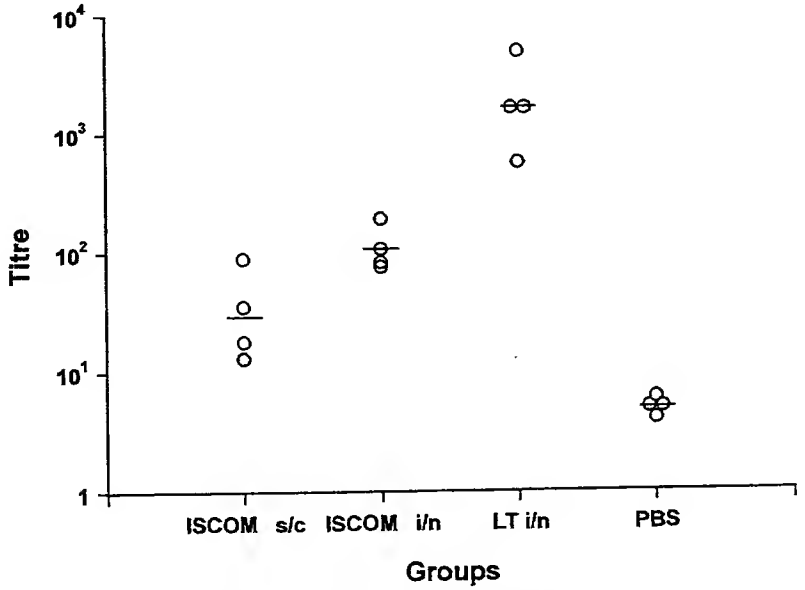

Fig. 5. Analysis of influenza specific IgA responses in faecal extracts after immunisation with the different experimental vaccines detailed in Table 2. Individual titres (O) and geometric mean titres (–) are shown after two immunisations.

only background levels of specific IgA were detected after subcutaneous immunisation with influenza-ISCOMs, which was not significantly different to control mice immunised with PBS.

The IgA response in faecal samples induced by intranasally administered influenza antigen mixed with LT was significantly higher than in the two ISCOM groups ($P < 0.05$). Slightly higher levels of influenza specific IgA in faecal samples were induced by influenza-ISCOMs given intranasally compared to subcutaneous administration (Fig. 5). This difference was not significant although only one out of four mice in the subcutaneous group had a titre comparable to the titres in the intranasal group. The response in the two ISCOM groups was significantly higher than in the control group given PBS ($P < 0.05$).

3.4. Cytotoxic T-lymphocyte response

A strong specific CTL response was induced in both the intranasally and subcutaneously immunised influenza-ISCOM groups (Fig. 6). Strong CTL responses against influenza infected target cells were also induced in the positive control group (data not shown). Mice in this group had recovered from challenge with influenza virus after a significant weight loss.

4. Discussion

In this study, we have shown that influenza-ISCOM vaccines, formulated at high doses of antigen and adjuvant and delivered intranasally, induced systemic humoral and cellular immune responses at similar levels to those seen following subcutaneous delivery and protected mice against live virus challenge. Furthermore, we have shown that the influenza-ISCOMs delivered intranasally induced mucosal immune responses equivalent to those obtained with the potent mucosal adjuvant LT. The intranasal influenza-ISCOMs contained 15 times the dose of both antigen and adjuvant as the subcutaneous influenza-ISCOMs. The increase in the dose was based on previous work in the laboratory, which had shown that the efficiency of subcutaneous ISCOM delivery was 15 times greater than mucosal delivery. The mechanism for this difference is not clear, but is likely to be due to inefficient uptake at mucosal surfaces.

Immunisation with the influenza-ISCOM formulations resulted in complete protection against challenge and the mice also gained weight irrespective of the delivery route. Furthermore, the dose of antigen required in the intranasal ISCOM group was over 30 times less than the amount required for complete protection with non-adjuvanted influenza. The protection levels following subcutaneous delivery of influenza-ISCOMs shown here are similar to previously published studies [19]. In contrast, lower protection rates have been reported following intranasal delivery of influenza-ISCOMs [10,12]. However, in these studies only the dose of antigen and not the adjuvant was increased in the ISCOM formulations. As the ISCOM provides both a delivery vehicle and immunodulatory capability, it is likely to be important to maintain the ratio of antigen and adjuvant to increase the efficiency of intranasal delivery.

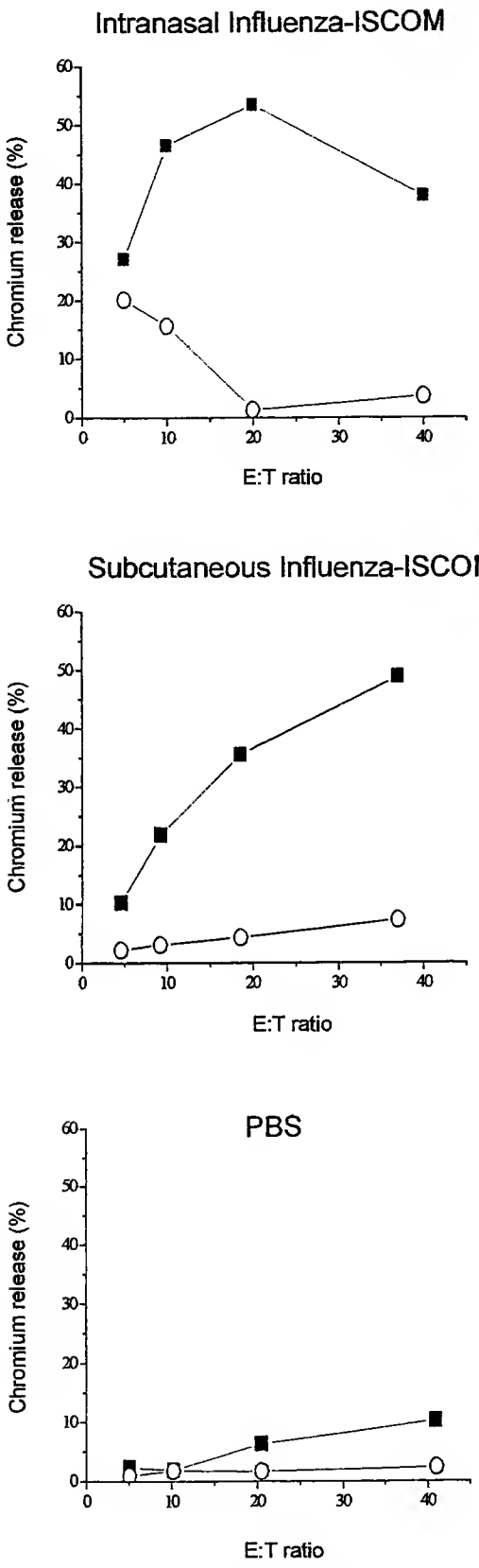

Fig. 6. Analysis of influenza specific splenic CTL responses. Mice were immunised with the different experimental vaccines detailed in Table 2. Spleen cells were incubated with A/PR8/34-influenza infected P815 cell (■, positive target) and non-infected P815 cells (○, negative target).

Induction of both systemic and mucosal antibody responses is a desirable characteristic of intranasally delivered vaccines. The presence of influenza specific serum IgG has been linked to prevention of disease progression in the deep respiratory tract after infection [21,22]. In this study, the serum IgG responses were equivalent for both the ISCOM formulations and the LT formulation, but serum IgA was significantly lower for the subcutaneously delivered ISCOMs, than either the intranasally delivered ISCOM or LT formulations. Generally lower levels of serum antibody have been reported following intranasal delivery of subunit vaccines compared to subcutaneous delivery, but again these studies have compared similar doses of vaccines delivered by the two routes [12,14,16]. Consistent with this little, if any, serum antibody was detected following intranasal immunisation with 5 μg of influenza antigen alone compared to good responses when given subcutaneously (data not shown).

Influenza specific mucosal IgA has been shown to correlate with prevention of virus infection and has been the major motivation for the development of mucosally delivered influenza vaccines [21,23]. The mucosal IgA responses for the intranasally delivered influenza-ISCOM and LT formulations were significantly higher than the subcutaneously delivered influenza-ISCOMs, where virtually no mucosal IgA response was detected. Although not done in this study, previous work in the laboratory has shown that no mucosal IgA responses are evident following vaccination with up to 10 μg of the influenza antigen alone. The induction of IgA in the gastrointestinal tract observed in this study was probably due to the mice swallowing some of the vaccine during immunisation.

LT has proven to be a potent mucosal adjuvant and although others have studied the use of ISCOM as a mucosal adjuvant previously, a comparison with LT has not been reported. Previous studies with ISCOM formulations have compared mucosal responses following intranasal and subcutaneous delivery but the differences have not been as high as those shown in the present study [17,24]. The intranasal doses used in those studies were five times greater than the subcutaneously delivered formulation, which would be sub optimal based on our data because they would not adequately compensate for the reduced efficiency of delivery by this route.

Cellular immune responses against influenza have been shown to correlate with recovery from infection [21]. Induction of CTL has been shown following intranasal immunisation with live influenza vaccines but very few studies have shown induction of CTL following intranasal immunisation with subunit vaccines [21,25,26]. In this study, CTL responses were induced following intranasal delivery of influenza-ISCOMs; these responses were similar in magnitude to those

induced with subcutaneously delivered influenza-ISCOMs. ISCOMs have been shown to be potent inducers of CTL following subcutaneous delivery with a range of antigens including influenza [10,27]. Induction of CTL in the spleen following intranasal immunisation of mice with an ISCOM formulation containing a measles CTL epitope has also been shown [13] but no studies to date have reported the induction of splenic CTL following intranasal delivery of influenza-ISCOMs. Induction of Th1 and Th2 immune responses is typical following subcutaneous immunisation with ISCOM and has also been shown following oral delivery [28,29]. We have found a similar pattern of response following intranasal delivery of influenza-ISCOM with induction of both splenic IFN-γ and IL-5 (Sjölander et al., unpublished data).

Efforts are currently underway to develop a mucosally effective human influenza vaccine. The intranasally delivered influenza-ISCOM vaccine described in this paper was protective against experimental infection, and induced both humoral and cellular immune responses including mucosal IgA, serum IgG and CTL. The results in this study indicate that development of a human intranasal influenza ISCOM vaccine warrants further study. Clearly one of the key issues to be addressed is the dose of antigen and whether intranasal delivery of an ISCOM-influenza vaccine can induce protective immune responses at a dose equal to or preferably below current vaccines. Intranasal delivery is also more likely to be acceptable in children where the current vaccines are considered unacceptable because they are to reactogenic. Furthermore, needless delivery is likely to enhance the use of influenza vaccines in the broader community.

Acknowledgements

The authors thank Charles Quinn, Dharma Picking, Christine Millar and Denise Fletcher for their excellent technical assistance. We are also grateful for the electron microscopy analysis performed by Ross Hamilton.

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